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Linear Structure of the Oligosaccharide Chains in α_1-Protease Inhibitor Isolated from Human Plasma*

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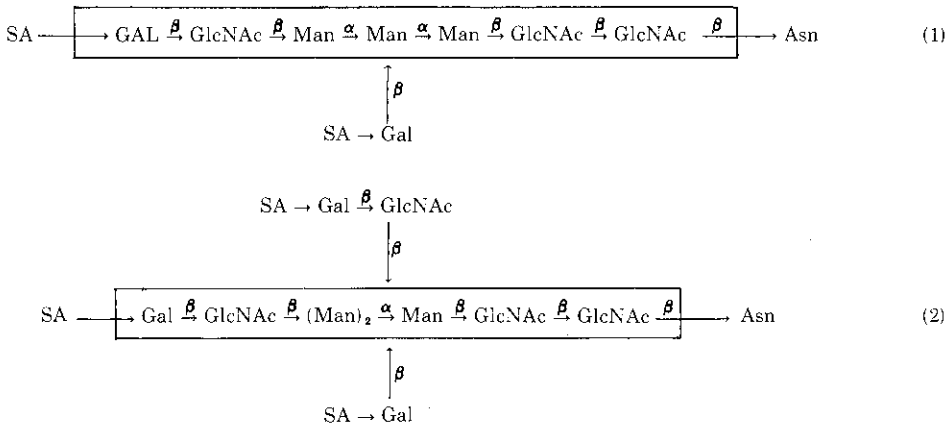
Two glycopeptides present in equal amounts were isolated from a pronase digest of α_1-protease inhibitor of human plasma by gel filtration on Sephadex G-50 and chromatography on DEAE-cellulose. The carbohydrate side chains in both glycopeptides are linked through asparaginyl residues. The glycopeptides were digested sequentially with specific glycosidases; and after each step, the released sugars as well as the composition of the residual peptides were determined. The linear structures of these glycopeptides deduced from these data are shown below. Based on the total carbohydrate content of the intact protein and with these structural data, it is postulated that 4 oligosaccharide units are attached to 1 molecule of the protein; 2 of these were represented as in Equation 1, the other 2 as in Equation 2.



(1)

(2)

Multiple genetic types of α_1-protease inhibitor (P_i, formerly α_1-antitrypsin) in human serum have been shown to be inherited by a series of autosomal codominant alleles that determine the concentration and the electrophoretic mobility of this protein (see Ref. 1, a review). Serum from individuals with low concentration of α_1-protease inhibitor (P_i type ZZ) usually contains only 10 to 40% as much of that protein as found in serum from individuals of the normal type (P_i type MM). This deficiency has been shown to be commonly associated with either an early onset of familial emphysema or familial infantile cirrhosis.

The α_1-protease inhibitor is a glycoprotein consisting of a single polypeptide chain (2, 3). Since the protein in P_i type ZZ has a slower mobility towards the anode in agarose gel electrophoresis than the corresponding protein of the P_i type MM (4), it is reasonable to postulate that net charge may be one of the chemical differences between the two types of protein. This difference may be due to amino acid substitutions in the polypeptide chain or to variations in the contents of sialic acid and the arrangement of the sugars in the carbohydrate moieties. Therefore, as a prelude to the investigation of the chemical differences between these two homologous pro-

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teins, it is important to establish the chemical structure of the normal protein (P_i type MM). The purpose of this paper is to report on the isolation of the glycopeptides from α_1-protease inhibitor after pronase digestion, and to propose the linear structures of the oligosaccharide chains based on data derived from enzymic studies.

MATERIALS AND METHODS

Isolation and Purification of α_1-Protease Inhibitor—α_1-Protease inhibitor was isolated and purified from outdated pooled human plasma (P_i type MM) according to previously described procedures (2) with two modifications. First, repeated chromatography on DEAE-cellulose was found to be unnecessary. The present procedure required chromatography on DEAE-cellulose only twice, once at pH 6.5, and then at pH 8.9. The remaining albumin was removed by affinity chromatography on blue dextran-Sepharose (5), prepared according to the method described by Ryan and Vestling (6). Secondly, the step of hydroxylapatite adsorption chromatography was also omitted. Instead, the protein was further purified by preparative electrophoresis on polyacrylamide gel (7) at pH 8.9. The final product inhibited trypsin on a mole to mole ratio (2). Data presented in Fig. 1 show the isolated protein to be homogeneous upon disc electrophoresis at pH 8.9 on polyacrylamide gel, to give a single precipitin band upon immunoelectrophoresis against rabbit antihuman serum, and to give a single band upon electrophoresis in the presence of sodium dodecyl sulfate and 2-mercaptoethanol.

Preparation of the Glycopeptide—A sample of α_1-protease inhibitor was dialyzed against 0.001 M sodium acetate buffer, pH 4.5, containing 6 M urea, for 24 hours and then heated to 70° for 1 hour. The denatured protein was dialyzed against 0.15 M Tris acetate, pH 8.0, containing 1.5 mM calcium acetate, and digested three times with pronase (2% by weight) for a total of 72 hours at 37°. The mixture was then chromatographed on a column (1.5 × 100 cm) of Sephadex G-50 (fine) equilibrated in 1 M acetic acid. Aliquots of the eluent were analyzed for carbohydrates by the orcinol method (10), and the absorbance was determined at 540 nm. In order to obtain glycopeptides with a minimal content of amino acids, the fractions containing carbohydrates were pooled, the pronase digestion was repeated, and the glycopeptides were reisolated. After dialysis, the glycopeptides were chromatographed on a column (0.5 × 30 cm) of DEAE-cellulose equilibrated in 0.004 M sodium phosphate buffer, pH 6.8. After washing the column with 30 ml of the same buffer, the chromatogram was developed with a linear gradient between 0.004 M (20 ml) and 0.10 M (20 ml) sodium phosphate buffer, pH 6.8. Molecular weights of the glycopeptides were estimated by using a column (0.9 × 180 cm) of Sephadex G-50 (superfine) equilibrated in 0.15 M NaCl solution, using blue dextran, raffinose, D-mannose, and glycopeptides isolated from fetuin and from ovalbumin as standards. The latter glycopeptides were prepared according to the same procedures described above for those from α_1-protease inhibitor. The average molecular weights of the isolated peptides were calculated based on their content of amino acids and carbohydrates and were similar to values reported in the literature (11, 12). They are 2898 and 1551, respectively, for fetuin and ovalbumin.

Chemical Analysis—Samples of the intact α_1-protease inhibitor and the glycopeptides were hydrolyzed under the following conditions: for amino acids, 6 N HCl at 110° *in vacuo* for 48 hours; for *N*-acetyl-D-glucosamine, 4 N HCl at 100° *in vacuo* for 6 hours; for neutral sugars, D-galactose, and D-mannose, 1 N H_2SO_4 in sealed tubes for 8 hours at 100°; and for sialic acid, 0.18 N H_2SO_4 at 80° for 1 hour. Amino acids and D-glucosamine were analyzed on a Technicon automated amino acid analyzer (13), and the neutral sugars were determined on an automated sugar analyzer according to Lee *et al.* (14). Sialic acid was determined according to the procedures described by Warren (15). Norleucine was used as an internal standard for the analysis of amino acids and D-glucosamine, and L-rhamnose and D-xylose were used for D-galactose and D-mannose. Preliminary experiments in which variations of time and temperature for hydrolysis of the glycopeptides and the glycoprotein were studied showed that the conditions described were the most satisfactory.

Oligosaccharide Structure by Enzymic Methods—All the exoglycosidases (β-*N*-acetylhexosaminidase, α-mannosidase, and β-mannosidase) were isolated and purified to the same degree of purity with the same specificity and similar specific activities as previously published (16, 17). Samples of glycopeptides were digested with 5% (w/w) neuraminidase (Sigma type VI, from *Clostridium perfringens*) in 0.05 M sodium citrate buffer, pH 5.0, at 37° for 16 hours. The asialoglycopeptides were reisolated by gel filtration on a column (1.5 × 100 cm) of Sephadex G-50 equilibrated in 1 M acetic acid. The composition of the asialoglycopeptides revealed the presence of less than 5% of sialic acid with respect to galactose, with little change in the composition of other carbohydrate constituents. Aliquots of the asialoglycopeptides were dried by evaporation, and the residues were dissolved in 0.05 M sodium citrate buffer, pH 4.0. These solutions were then incubated with one or more appropriate glycosidases at 37° for 48 hours. After incubation, aliquots of the reaction mixtures were placed in an automated sugar analyzer for the determination of liberated D-mannose and D-galactose. Liberated *N*-acetyl-D-glucosamine was determined by the Morgan-Elson reaction as described by Reissig *et al.* (18). Residual glycopeptides were reisolated by gel filtration on a Bio-Gel P-2 column (0.5 × 60 cm) equilibrated in water, and the contents were analyzed after hydrolysis as described above. This is essentially the "subtractive method" of sequence determination commonly used in protein/peptide chemistry.

NH_2- and COOH-terminal Residues of α_1-Protease Inhibitor—The NH_2-terminal residue of α_1-protease inhibitor was determined by the Sanger method (19), by digestion with diisopropyl fluorophosphate-treated leucine-aminopeptidase (20), and by the dansylation[1] method (21). The COOH-terminal residues were identified in an amino acid

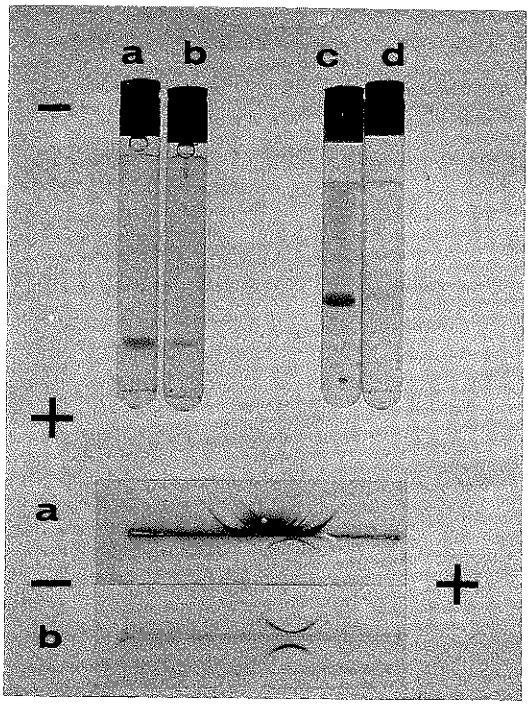

FIG. 1. Purified α_1-protease inhibitor on disc electrophoresis, sodium dodecyl sulfate electrophoresis (*top*) and upon immunoelectrophoresis (*bottom*). Disc electrophoresis: samples of purified α_1-protease inhibitor (20 μg) were subjected to disc electrophoresis on polyacrylamide gel at pH 8.9. One gel (*a*) was stained with Coomassie blue; another (*b*) was stained with periodic acid/Schiff reagents (8). Sodium dodecyl sulfate electrophoresis: samples of purified α_1-protease inhibitor (20 μg) were subjected to electrophoresis on polyacrylamide gel in the presence of 2-mercaptoethanol and detergent according to published procedures (9). One gel (*c*) and another gel (*d*) were stained with Coomassie blue and periodic acid/Schiff reagents, respectively. Immunoelectrophoresis: samples of normal human serum (5 μl) and purified α_1-protease inhibitor (10 μg) were placed in the *top* and *bottom wells*, respectively, on both *slides*. After electrophoresis in 0.1 M sodium barbital buffer, pH 8.0 for 90 min at 200 volts, rabbit antihuman serum and goat antiprotease inhibitor (Kallested Co.) were placed in the *center troughs* of *slides a* and *b*, respectively. Diffusion was allowed to proceed for 16 hours. The gels were washed, dried, and stained with Amido black.

[1] The abbreviation used is: dansyl, 5-dimethyaminonaphthalene-1-sulfonyl.

analyzer after digestion with diisopropyl fluorophosphate-treated carboxypeptidase A (22).

RESULTS

Chemical Compositions, NH_2- and COOH-terminal Residues of α_1-Protease Inhibitor—For all calculations, the molecular weight of the protein was assumed to be 54,000. The extinction cooefficient $E_{280}^{1\,mg/ml}$ of the purified α_1-protease inhibitor was calculated to be 0.579. The amino acid composition of the protein was shown to be essentially the same as that previously reported (2) and is not shown here. The composition of the carbohydrates per mol of α_1-protease inhibitor is: D-mannose, 12; D-galactose, 9; *N*-acetyl-D-glucosamine, 14; and sialic acid, 9. No fucose was detected. On a weight basis, the carbohydrate content of the protein is 16.4%, which compares well with the value of 17.2% calculated based on the proposed structures of the oligosaccharide units presented in this communication. These values are in good agreement with those reported by Kress and Laskowski (23) and Pannell *et al.* (24), if, in the latter case, the number of sialic acid residues are excluded in the calculations. They are considerably higher, however, than those reported by Heimburger *et al.* (25) and Crawford (3). The NH_2-terminal residue of α_1-protease inhibitor is probably blocked, since repeated attempts using conventional methods were unsuccessful in identifying any NH_2-terminal residue. The COOH-terminal residue is either leucine or phenylalanine, because both amino acids were released in equal amounts when the protein was digested with carboxypeptidase A.

Isolation and Chemical Composition of Glycopeptides—A sample of α_1-protease inhibitor (60 mg) was digested with pronase, and the mixture was chromatographed on Sephadex G-50 as shown in Fig. 2. There are two zones containing carbohydrates: the first represents either the intact protein or a group of large glycopeptides due to incomplete digestion; the second represents the small glycopeptides. Of the total carbohydrate applied to the column, over 90% was present in these two zones. When the fractions pooled from the first peak were again digested with pronase and rechromatographed on the same column, only the second peak containing the smaller glycopeptides was obtained. The fractions containing the smaller glycopeptides from both column runs (designated as P-1) were combined and further digested with pronase, and the glycopeptide fraction (designated as P-2) was similarly reisolated. The compositions in amino acids and carbohydrates of glycopeptide fractions P-1 and P-2 are shown in Table I. Aspartic acid is the major amino acid present, while the others, threonine, serine, glutamic acid, glycine, and alanine, are the minor amino acids. After the second round of pronase digestion, the amino acid composition of the glycopeptides (P-2) showed a decrease in the content of the latter five amino acids, while that of aspartic acid was not affected. These data indicate that the carbohydrate moiety in α_1-protease inhibitor is attached to asparagine.

To determine whether there was more than one glycopeptide species in this fraction, P-2 was chromatographed on DEAE-cellulose and developed with an iso-pH linear gradient of increasing concentration of sodium phosphate. The elution profile is shown in Fig. 3. Over 80% of the constituent carbohydrate was recovered in three zones (I, II, and III). The first zone contained less than 5% of the total recovered mannose, while the second and the third zones contained 46% and 49%, respectively. The chemical composition of these resolved glycopeptides is also shown in Table I. The differences between glycopeptides II and III exist in the content of sialic acid, *N*-acetyl-D-glucosamine, and -D-galactose. On the other hand, all the resolved peptides contained the same molar ratio of D-mannose. The amino acid compositions of the two glycopeptides also show a difference. The major amino acid in

TABLE I

Carbohydrate and amino acid compositions of glycopeptides

Compositions are given as molar ratios. Numbers in parentheses are the assumed stoichiometric numbers of residues per mol of peptide. See under "Materials and Methods" for other details.

	P-1	P-2	I	II	III
Sialic acid	2.2	N.D.[a]	1.0	2.2 (2)	3.2 (3)
D-Galactose	2.4	2.4	3.1	1.9 (2)	2.8 (3)
D-Mannose	3.0	3.1	2.7	2.9 (3)	2.9 (3)
N-Acetyl-D-glucosamine	3.5	3.4	3.8	2.9 (3)	3.9 (4)
Aspartic acid	1.4	1.1	1.4	1.1 (1)	1.2 (1)
Threonine	0.5	0.2	0.5	<0.1	0.6 (1)
Serine	0.9	0.5	0.9	0.1	1.1 (1)
Glutamic acid	0.4	0.1	0.3	<0.1	0.2
Glycine	1.5	0.4	1.0	0.9 (1)	0.4
Alanine	0.3	<0.1	0.4	<0.1	0.1
Leucine	0.2	<0.1	0.3	<0.1	<0.1

[a] N.D., not determined.

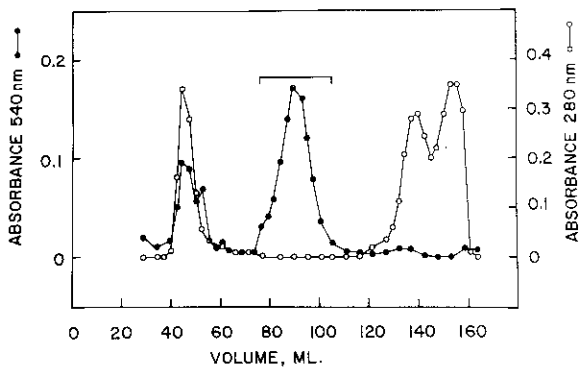

FIG. 2. Gel filtration of a pronase digest of α_1-protease inhibitor on Sephadex G-50. The flow rate was 0.8 ml/min. The fractions which were pooled were marked by a *horizontal bar*. See under "Materials and Methods" for other details.

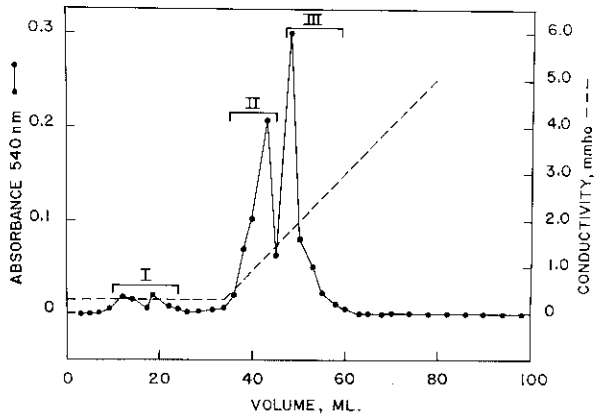

FIG. 3. DE52 chromatography of glycopeptides. Flow rate was 0.24 ml/min. The *horizontal bars* indicate the fractions which were pooled. See under "Materials and Methods" for other details.

addition to aspartic acid in glycopeptide II is glycine, while in glycopeptide III they are threonine and serine. Glycopeptide I, on the other hand, appears to be the partially desialated product of glycopeptide III, with a longer peptide moiety.

Structural Determination of Glycopeptides II and III—Because glycopeptide I is present in such a small amount, no further structural work on it was undertaken. The linear structures of glycopeptides II and III were established by enzymic methods. These studies are summarized in Tables II, III, and IV. Data in Table II show that all the constituent carbohydrates in asialoglycopeptide Fraction P-2 can be released completely by digestion in the presence of the following specific enzymes: β-galactosidase, β-*N*-acetylhexosaminidase, and α- and β-mannosidase. These enzymes were therefore used for the structural determination of glycopeptides II and III. Also, these data established that the conditions used in these enzymic studies were optimal for the release of sugars.

Without prior removal of the sialic acid residues, neither glycopeptide II nor III was susceptible to digestion with any of the glycosidases. Similarly, the asialoglycopeptides II and III were not susceptible to digestion with β-*N*-acetylhexosaminidase, α- and β-mannosidase. On the other hand, all D-galactose residues, 2 and 3, respectively, present in asialoglycopeptides II and III were released by the action of β-galactosidase. The corresponding residual peptides B contained no detectable amount of D-galactose, while the content of the other carbohydrate moieties remained essentially unchanged. These data demonstrate that the sequence sialic acid→D-galactose occupies the nonreducing terminal end of the oligosaccharide units in both of the glycopeptides. Since there is more than one sialic acid and D-galactose residue present in these peptides, the results do not distinguish whether these residues are arranged in linear array or derived from several branched chains. If the latter arrangement proves to be the case, glycopeptides II and III must consist of two and three nonreducing terminal branched chains, respectively, having the structure sialic acid→D-galactose. The branched chain structures are analogous to those established structures for oligosaccharide units derived from many other plasma proteins (12).

When the asialoglycopeptides (residual peptides A) II and III were digested with β-galactosidase and β-*N*-acetylhexosaminidase, in addition to the expected release of D-galactose, 1 and 2 residues of *N*-acetyl-D-glucosamine, respectively, were released. The composition of the two residual peptides (residual C), after this step of digestion, were shown to be identical; each consisting of 3 residues of D-mannose, and 2 residues of *N*-acetyl-D-glucosamine. These core *N*-acetyl-D-glucosamine residues were not released until all the D-mannose residues had been removed by α- and β-mannosidase. When residual peptides C derived from glycopeptides II and III were digested with β-mannosidase, no appreciable release of sugars was observed. However, digestion with α-mannosidase, in both cases, removed 2 residues of D-mannose from these residual peptides, and the release of the last D-mannose residue was observed only upon digestion with both α- and β-mannosidase. These data indicate that the 2 α-mannose residues are positioned at the nonreducing terminal end of the residual peptides C and that the β-mannose residue is internal to an α-mannose residue. It is

TABLE II

Preliminary experiments on glycosidase digestion of asialoglycopeptide P-2

Asialoglycopeptide P-2 used in this experiment was obtained from a pronase digest different from that reported in Table I. The composition in sugars is similar, but not identical, to that reported in Table I. Aliquots of P-2 (11 nmol in 50 μl) were digested with glycosidases, and the released sugars were measured as described under "Materials and Methods."

Enzyme digestion	Sugar released		
	D-Mannose	D-Galactose	*N*-Acetyl-D-glucosamine
	nmol		
Acid hydrolysis	30.1	25.0	39.0
β-*N*-Acetylhexosaminidase + α- and β-mannosidase	<3	<3	<3
β-Galactosidase	<3	25.2	<3
β-Galactosidase + β-*N*-acetylhexosaminidase	<3	25.2	14.2
β-Galactosidase + α-mannosidase	<3	25.2	<3
β-Galactosidase + β-mannosidase	<3	25.2	<3
β-Galactosidase + β-*N*-acetylhexosaminidase + α-mannosidase	19.4	25.0	15.0
β-Galactosidase + β-*N*-acetylhexosaminidase + β-mannosidase	<3	25.0	15.0
β-Galactosidase + β-*N*-acetylhexosaminidase + α- and β-mannosidase	30	25.0	39.0

TABLE III

Glycosidase digestion of glycopeptide II

The released sugars and the composition of the residual peptides after enzymic digestion are reported as molar ratios. The numbers in parentheses are the assumed stoichiometric ratios of the constituents in the peptides. See under "Materials and Methods" for other details.

	Glycopeptide II + neuraminidase		Residual A + β-galactosidase		Residual A + β-galactosidase + β-*N*-acetylhexosaminidase		Residual C + α-mannosidase		Residual C + α- and β-mannosidase		Residual E + β-*N*-acetylhexosaminidase	
	Released sugar	Residual A	Released sugar	Residual B	Released sugar	Residual C	Released sugar	Residual D	Released sugar	Residual E	Released sugar	Residual F
Sialic acid	N.D.[a]	<0.1	N.D.	ND	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
D-Galactose	N.D.	1.9 (2)	2.0 (2)	<0.1	1.9 (2)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	N.D.
D-Mannose	N.D.	2.9 (3)	<0.1	3.1 (3)	<0.1	3.2 (3)	2.1 (2)	1.1 (1)	2.9 (3)	0.2	<0.1	N.D.
N-Acetyl-D-glucosamine	N.D.	3.2 (3)	<0.1	2.9 (3)	1.1 (1)	1.7 (2)	<0.1	1.7 (2)	0.1	1.8 (2)	1.8 (2)	N.D.
Aspartic acid	N.D.	1.0 (1)	N.D.	1.0 (1)	N.D.	1.1 (1)	N.D.	1.2 (1)	N.D.	1.0 (1)	N.D.	N.D.

[a] N.D., not determined.

TABLE IV

Glycosidase digestion of glycopeptide III

See legend to Table III and under "Materials and Methods" for other details.

	Glycopeptide III + neuraminidase		Residual A + β-galactosidase		Residual A + β-galactosidase + β-*N*-acetyl-hexosaminidase		Residual C + α-mannosidase		Residual C + α- and β-mannosidase	
	Released sugar	Residual A	Released sugar	Residual B	Released sugar	Residual C	Released sugar	Residual D	Released sugar	Residual E
Sialic acid	N.D.[a]	<0.1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
D-Galactose	N.D.	2.8 (3)	3.1 (3)	<0.1	3.0 (3)	0.1	<0.1	<0.1	<0.1	<0.1
D-Mannose	N.D.	3.1 (3)	<0.1	3.0 (3)	<0.1	3.1 (3)	1.5 (2)	1.5 (1)	1.6	1.5
N-Acetyl-D-glucosamine	N.D.	3.8 (4)	<0.1	N.D.	1.8 (2)	1.7 (2)	0.1	2.3 (2)	0.1	1.7 (2)
Aspartic acid	N.D.	1.3 (1)	N.D.	1.1 (1)	N.D.	1.1 (1)	N.D.	1.2 (1)	N.D.	1.2 (1)

[a] N.D., not determined.

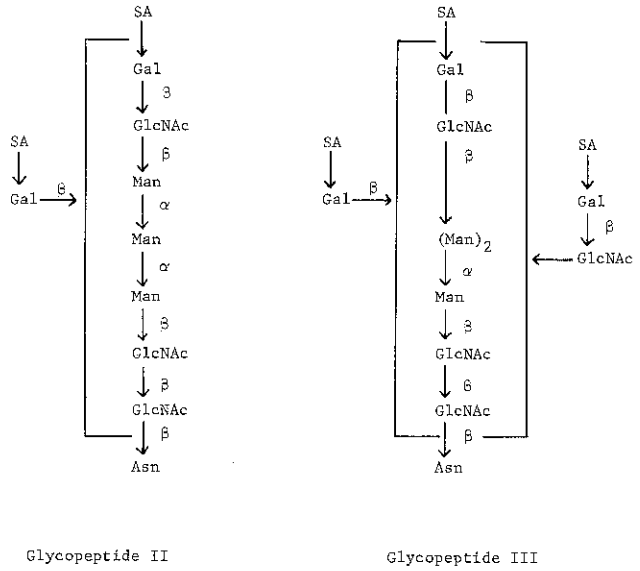

FIG. 4. Proposed structures of glycopeptides II and III. *SA*, sialic acid.

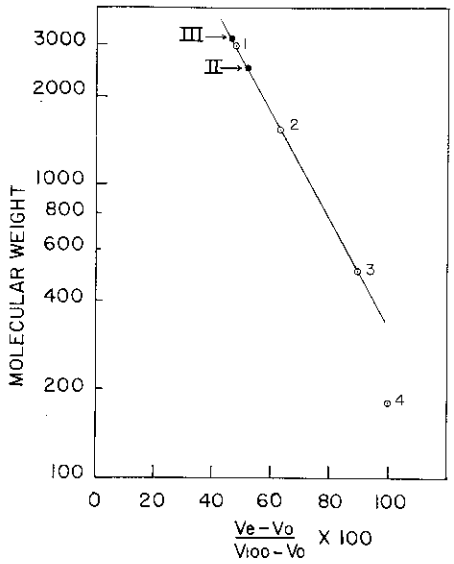

FIG. 5. Molecular weight determination of glycopeptides II and III by gel filtration on Sephadex G-50. The flow rate was 0.17 ml/min, and 1.5-ml fractions were collected. The samples contained, in addition to either glycopeptide II or III, the following molecular markers: blue dextran to locate the void volume (V_0); D-mannose to determine V_{100}, designated as *4;* raffinose, (M_r = 504) designated as *3;* an ovalbumin glycopeptide, designated as *2;* a fetuin glycopeptide, designated as *1*.

noted that while the data obtained for glycopeptide II (Table III) are indeed unequivocal in supporting our conclusion, the data presented in Table IV for glycopeptide III on this point are ambiguous. It appears that the digestion of residual peptide C with the mannosidases was incomplete, probably due to the presence of partially inactivated α-mannosidase. However, our conclusion is consistent with data obtained for glycopeptide P-2 (see Table II), from which glycopeptides II and III are derived. Finally, the residual peptide E derived from glycopeptide II (Table III), consisting of 2 core residues of *N*-acetyl-D-glucosamine and 1 residue of aspartic acid, was shown to release both *N*-acetyl-D-glucosamine residues upon digestion with β-*N*-acetylhexosaminidase, firmly establishing the core structure to be $GlcNAc^{\beta}\rightarrow GlcNAc^{\beta}\rightarrow Asn$. Thus, the enzymic data herein presented are consistent with the postulated structures of glycopeptides II and III as shown in Fig. 4. Since the enzymic data do not determine branching points or the position of glycosidic linkage, the postulated structures do not provide this information. Based on the compositions listed in Table I, the molecular weights of glycopeptides II and III (including both carbohydrates and amino acids) are calculated to be 2188 and 2977, respectively. When these glycopeptides were chromatographed on Sephadex G-50 (superfine) along with known glycopeptide markers, the molecular weights of glycopeptides II and III (Fig. 5) were determined to be 2500 and 3200, respectively. These results are in reasonable agreement with the calculated figures based on the composition data and are consistent with the postulated structures.

DISCUSSION

The results reported in this communication establish that the oligosaccharide units in α_1-protease inhibitor are linked to asparagine residues and are composed of complex heteropolysaccharides. The 2 oligosaccharide units (Fig. 4) probably differ in the number of branched chains. The amino acid residues adjacent to the asparagine residues to which the carbohydrate units are attached appear also to be unique for each unit and are probably derived from different areas of the polypeptide chain.

It has been demonstrated that glycopeptides II and III are present in equal amounts in the α_1-protease inhibitor. Based on the total content (16.4%) of the carbohydrates in the intact

protein, it was calculated that 4 oligosaccharide units were attached to 1 molecule of the protein: 2 of these were represented as in glycopeptide II, the other 2 as in glycopeptide III.

It has been reported (26) that a protein in the liver plasma membrane preferentially binds asialoglycoproteins. Therefore, it can be assumed that any circulating glycoprotein which is not fully sialated must be degraded rapidly. In the present study of α_1-protease inhibitor, both glycopeptides II and III were isolated in fully sialated forms and only glycopeptide I, representing less than 5% of the total isolated glycopeptides, can be partially desialated. Hence, it is concluded that normal α_1-protease inhibitor is present almost entirely in a fully sialated form, with little or no exposed galactosyl termini. This conclusion is consistent with the reported half-life (6 days) observed (27) for the normal circulating α_1-protease inhibitor. Consequently, these data cannot be reconciled with the report of Pannell *et al.* (24) which claimed that human α_1-protease inhibitor contains 21 residues of hexose but only 2 residues of sialic acid per mol of protein.

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